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(54) Title: RECOVERY OF PRODUCTS FROM PLANT ROOTS

(57) Abstract

Intact living plants or plant parts are treated to increase production of at least one chemical compound in the roots of the plant. The roots are harvested and the chemical compound(s) is recovered from the roots.

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RECOVERY OF PRODUCTS FROM PLANT ROOTS

This invention relates to the recovery of chemical products from plants.

Plants have long been recognized as being a potential source of chemical products (phytochemicals) and, to date, a wide variety of compounds of commercial interest, including those having pharmaceutical activity, have been recovered from plants. In general, such compounds have been recovered and used either as a crude extract or as purified compounds which require the use of complex extraction and purification procedures.

For example, genistein and daidzein are isoflavonoids present in a number of plants which have been recognized as having anti-cancer activity. To date, however, such chemicals are available only to the consumer in the form of soybean flours and other crude soybean products.

Other plant chemicals are available in purified forms; however, they are generally recovered only after a costly and laborious tissue extraction procedure.

In one aspect, the present invention is directed to recovering chemical compounds from plants by a new procedure.

In another aspect, the present invention is directed to screening of plants for potential compounds of interest by recovering and isolating such compounds from plants by a new and improved procedure.

In accordance with an aspect of the invention, a living plant or plant portion is subjected to treatment and/or conditions to induce and/or increase production of one or more compounds in the roots of the plant, followed by harvesting of roots from the plant and recovering from the roots compound(s) produced or increased in quantity as a result of the treatment.

In a preferred embodiment, the plant which is treated and from which the roots are recovered is hydroponically or aeroponically produced.

In accordance with a particularly preferred embodiment, the roots are harvested in a manner such that the plant remains alive and can grow new roots for future harvesting and recovery of a compound(s).

In accordance with another aspect of the present invention, there is generated a chemical compound library which may be used for screening for a desired compound or activity. In this respect, plants or plant parts which are specifically grown or maintained for the purpose of recovering compounds therefrom are subjected to treatment while alive in order to induce in the roots a variety of compounds for potential screening. The roots are harvested and compounds are recovered therefrom for screening.

In accordance with still a further aspect of the invention, the process of the present invention may be employed for commercial production of desired compounds by specifically growing and maintaining plants or plant parts for recovery of desired compounds from the roots by inducing production in the roots of a new compound(s) or increased production of a compound(s).

DESCRIPTION OF DRAWINGS

Figure 1 is an HPLC profile of the diversity of compounds recovered from Solanum melongena without treating with an elicitor;

Figure 2 is an HPLC profile of the diversity of compounds recovered from root extracts from Solanum melongena which was treated with an elicitor;

Figure 3 is an HPLC profile of the diversity of compounds recovered from Daucus carota which was treated with an elicitor;

Figure 4 is an HPLC profile of the diversity of compounds recovered from Glycyne mad which was treated with elicitors, as compared to a control;

Figure 5 is an HPLC profile of the diversity of compounds recovered from Daucus caroto which was treated with elicitors, as compared to a control;

Figure 6 is an HPLC profile of the diversity of compounds recovered from Daucus caroto which was treated with elicitors, as compared to a control;

Figure 7 is an HPLC profile of the diversity of compounds recovered from Lycopersicon aesculentum which was treated with elicitors, as compared to a control; and

Figure 8 is an HPLC profile of the diversity of compounds recovered from Lupinus polyphyllus which was treated with elicitors, as compared to a control.

The plant or plant portion (which plant portion may or may not be the plant roots) which is treated to induce production or increased production of one or more compounds in the roots thereof is a living plant or plant portion which is intact, and which is capable of being sustained without the use of organic nutritional supplements and without maintaining sterile conditions for the plant. In accordance with the present invention, however, inorganic supplements may be employed in order to increase plant growth.

The plant which is used in the present invention may be an entire grown plant or a plant seed or seedling or a plant shoot or root, provided that the plant or plant portion is intact, alive and is capable of being maintained without an organic nutritional supplement, and without maintaining sterile conditions for the plant.

The living plant or plant part is contacted with water and in a preferred embodiment, at least the root of the plant is contacted with the water.

The contact with water may be effected by placing at least the plant roots in water or by "aeroponics," which involves contacting the plant, in particular, the roots of the plant, with water droplets from which chemical compound(s) is recovered.

The intact living plant or plant part which is subject to the process of the invention may be in the form of an entire plant or plant seedlings or seeds or plant roots, and, in each case, the plant or plant part is contacted with water, which may be pure water or water containing appropriate additives.

In accordance with a preferred aspect, the chemical product(s) are those which are recovered from the roots harvested from a plant which has been treated with an elicitor or inducer to increase production in the plant roots of one or more products. The inducer or elicitor may be in the water which contacts the plant or may be separately applied to the plant.

In one such embodiment, the plant or rooted shoots may be grown hydroponically and, in such a case, the plants are cultivated on top of a mesh with a small portion of their root system anchored in a layer of artificial soil above the mesh. This artificial soil layer is employed to supply all essential nutrients to the plants. In such an embodiment, a major portion of the root system grows through the mesh and soil layer into water which is below the mesh layer. The elicitor may be included in the water.

In a further embodiment, plants may be germinated and supported in rockwool cubes, as known in the art, with roots extending into a water solution which contains inorganic nutrients. The water may contain an elicitor.

Although in a preferred embodiment, the process is a continuous process, a batch process may also be employed.

The plants which are used in the invention may be any one of a wide variety of plants and may be sexually or vegetatively propagated plants.

The harvested roots may then be treated to recover compounds therefrom; eg., by solvent extraction.

Chemicals which are recovered from the extraction media, for example, by chromatography, may then be analyzed by various techniques in order to assist in identifying the recovered compounds. Such procedures are known in the art and should be apparent to those skilled in the art from the teachings herein.

As hereinabove indicated, the plants or plant parts which are subjected to the present invention are alive and intact, and in using a whole plant, the process does not destroy the plant; i.e., the plant is capable of continuing its growth. Moreover, the plants are capable of being grown and maintained without any organic supplements (although an organic supplement could be used if desired) and, therefore, are different than plants or plant parts which are cultured in a laboratory in that such cultured plants or plant parts require organic nutritional supplements and sterile conditions in order to maintain growth. Thus, in accordance with the present invention, the plant is grown and maintained in a growth state similar to that in a natural surrounding, and the plant functions as a natural bioreactor for producing valuable plant products in the roots as a result of treatment to induce or increase production of chemicals in the roots.

As hereinabove indicated, in accordance with an embodiment, the plants or plant parts maybe subjected to physical or chemical treatment to elicit or induce an increased production of one or more compounds. The plants, in particular roots thereof, may be contacted with an elicitor or inducer, which is a chemical compound, for example, organic and inorganic acids, fatty acids, glycerides, phospholipids, glycolipids, organic solvents, amino acids and peptides, monosaccharides, oligosaccharides, polysaccharides and lipopolysaccharides, phenolics, alkaloids, terpenes and terpenoids, antibiotics, detergents, polyamines, peroxides, ionophores, etc., or subjected to a physical treatment, such as ultra-violet radiation, low and high temperature stress, osmotic stress induced by salt or sugars, nutritional stress defined as

depriving the plant of essential nutrients (N, P, or K), in order to induce or elicit increased production of one or more chemicals. Such chemical compound or physical treatment may be applied continuously or intermittently to the plant or plant part. In one embodiment, such treatment may be accomplished by contacting the plant roots with a solution containing the elicitor or by irradiating the roots or exposing them to other environmental stresses such as temperature stresses; however, the invention is not limited to such an embodiment in that other portions of a plant or seedlings may be contacted with an elicitor.

The present invention may be employed for screening for potentially valuable products. In one embodiment, the plant or plant part may be subjected to these treatments, and chemical compounds produced by the plant may be recovered from the roots as hereinabove described. The recovered phytochemicals may be screened to ascertain whether treating of the plant produces a potentially valuable chemical compound.

The present invention will be further described with respect to the following examples; however, the scope of the invention is not to be limited thereby:

Example 1. Plant Production.

Seeds were germinated in a greenhouse equipped with supplementary lighting (16-h photoperiod 24-28°C). Seeds were placed inside 0.9 cm diameter, 0.9 cm deep well drilled in Grodan rockwool cubes (3.4 cm width x 3.4 cm depth x 3.7 cm height) purchased from Grodania A/S, Hedehousene, Denmark.

Depending on the speed of germination, the seeds were either placed directly into the rockwool cubes or sterilized to prevent rotting during the germination process. For sterilization, seeds were immersed first in 70% ethyl-Alcohol for 10-15 seconds, then in 2.5% Sodium Hypochlorite for 10-15 min., and finally rinsed thoroughly with distilled water. The sterilized seeds were placed in a Petri dish lined with no. 1 Wattman paper (Wattman International Ltd., Maidstone, England), soaked in either

a sterile water for seeds larger than 1 mm in diameter, or for smaller seeds with mineral salts nutrient solution. The Petri-dishes were sealed with parafilm before being placed in a growth chamber (12-h photoperiod 22-24°C) until the seeds germinated.

Rockwool cubes were placed inside standard greenhouse plastic trays (dimensions 52 cm width x 25 cm depth x 7 cm height) and watered with an intermittently operating overhead misting system triggered by a moisture sensor (Mist-A-Matic, E.C. Geiger Inc., Larleysville, PA). Seeds were allowed to germinate for 3-6 days till the roots started to emerge from the bottom of the rockwool cube.

After germination, the cubes with the seedlings were inserted into a 3.2 cm diameter round opening cut in the center of Styrofoam ring (8.2 cm diameter, 2.5 cm thickness). The ring was floated on the surface of 400-800 mL of hydroponic nutrient solution (2 g/L Hydro-Sol [Scotts-Sierra Horticultural Products Comp., Marysville, OH] supplemented with 1.2 g/L $Ca[NO_3]_2$) containing inside light impermeable, high-density polyethylene cylinder (9.0 cm in diameter, 16 cm in height).

Aeration was provided either by shaking the cylinders at 50 rpm on the platform shaker (Model Orbit, Lab-Line Instruments, Inc., Melrose Park, IL) or by bubbling compressed air through the solution. Seedlings were cultivated hydroponically in this system for 3 to 5 weeks with roots growing in a nutrient solution. Thereafter, the root system (average root dry weight 0.1±0.05g) was removed from the nutrient solution and placed inside a 30 mL glass beaker, containing 10-20 mL of distilled water or distilled water supplemented with the elicitor. To prevent the water loss from the plant canopy and the drying of the collecting solution, shoots of the plants were covered with a plastic bag. In the case where an elicitor is used, the plant was treated with the elicitor 24 hours prior to harvesting of the roots.

Example 2. Root Extraction

The complete root systems of all plants from each tray were excised, drained and weighed. Up to 30 g of the root systems (fresh weight) were sampled and stored at -20°C. The root tissue was homogenized in a laboratory blender (Model 31BL91, Waring, New Hartford, CT) in 2 volumes H_2O for approximately 30 sec. The homogenate was transferred to a 150 ml Corex tube (Corning, Inc., Corning, NY) and a two-phase extraction was carried out by adding 2 volumes of ethyl acetate (EA) and shaking the sealed tube for 30 min. at 200 rpm (Shaker Model PR70, Hoefer Scientific Instrument, San Francisco, CA). The tubes were then centrifuged (Model Avanti J-25, Rotor No. JA-14, Beckman Instrument Inc., Palo Alto, CA) for 10 min. at 4000 x g, in order to form a clear EA layer in the upper phase. The two-phase extraction was repeated with an additional single volume of EA. Following two extractions, the EA extracts were combined and placed in the fume hood until the EA volume was reduced to approximately half. The EA extract was divided into disposable glass tubes in proportion to the weight of the extracted roots, so that each tube contained the extracts equivalent to at least 5 g root tissue. The EA extract was evaporated in a speed vac (Model AES2010, Savant Instruments, Inc., Farmingdale, NY), the tubes were sealed and stored at -20°C. The H_2O phase, containing the root tissue and some EA residues, was filtered, pressed through a 70 μm nylon mesh (Spectra/Mesh Nylon Filters, Spectrum, Houston, TX) and placed in a 125 ml separatory funnel until the lower water phase separated from the upper layer (approximately 30 min). The water layer was decanted into 50 ml polypropylene disposable tubes and centrifuged for 30 min. at 4000 x g (Rotor No. JS-4.0, Beckman Instrument Inc.). The supernatant was divided into 60 ml glass bottles in proportion to the weight of the extracted root tissue (extract equivalent of 5 g of root tissue per bottle), freeze dried overnight (Genesis SQ12, Virtis, Gardiner, NY) and stored at -20°C. The remaining root tissue was further extracted with 2 volumes MeOH/CH_2Cl_2 (1:3), shaken for 30 min at 200 rpm,

filtered and pressed through 70 μm fluorocarbon mesh (Spectra/Mesh Fluorocarbon Filters, Spectrum). The filtrate was transferred to a separatory funnel until the lower MeOH/CH_2Cl_2 phase became clear (up to 30 minutes). The MeOH/CH_2Cl_2 extract was then divided into disposable glass tubes (equivalent of 5 g root tissue per tube), dried in a speed vac and stored in a similar manner to the EA extracts.

Example 3. High-pressure liquid chromatography (HPLC) analysis of extracted products

The chromatography separation of extracted products was performed with an HPLC-system consisting of Waters 996 Photodiode Array Detector (PDA) with usable UV range from 190 to 800 nm; a Waters 717 plus autosampler; two Beckman 110B solvent Delivery Modules, connected with a Beckman System Organizer (mixer) and a Beckman System Gold Analog Interface Module 406. The Beckman solvent delivery system was controlled by a NEC PC-8300 computer. Chromatography and spectral data was managed by Waters Millennium chromatography software, version 2.10, using a NEC Image 466es computer. All hardware components, except the solvent delivery system, were connected through a standard IEEE communication system. Compounds were separated on a Waters Nova Pak® C-18 reverse phase column, 3.9 x 150 mm, 60Å pore size, and 4 μm particle size.

The mobile phase consisted of two components: Solvent A - 0.5% ACS grade acetic acid in double distilled water, pH 3-3.5; and Solvent B - acetonitrile. Prior to use, each batch of solvent A was degased under vacuum and ultrasonication for 5 minutes.

The mobile phase flow was adjusted to 1 ml/min, and a gradient mode of separation was used for all separations. The gradient profile was as follows:

0 - 20 min 0% B - 100% B;

20 - 22 min 100% B;

22 - 25 min 100% B - 0% B;

25 - 33 min 0% B (column equilibration for next injection).

Compounds were detected with PDA detector within the wavelength range of 200 to 400 nm or with Waters Thermabeam™ Mass Detector. The column temperature was ambient.

All plants were grown hydroponically and treated with an elicitor, as described in Example 1. The roots were harvested and subjected to an extraction procedure as described in Example 2. The accompanying drawings are HPLC profiles (obtained as in Example 3) of chemicals recovered from the extracts, which extracts are recovered from roots harvested from the plants treated with elicitors described in the drawings.

Thus, in accordance with the present invention, plants or plant parts may be grown on a large scale and used to effectively generate a diverse library of compounds for screening for various applications. Such diversity may be obtained by using a variety of plants and a variety of elicitors. Such library may be effectively generated and screened in that such variety of compounds may be recovered from plant roots.

Furthermore, the present invention provides a "factory" for large scale production of compounds in that desired compounds can be simply recovered from roots of plants which are hydroponically grown. Moreover, by use of a selected elicitor, desired compounds which may not be normally present in the roots or which may not be present in sufficient quantities in the roots can be recovered on a large scale from roots of hydroponically grown plants by harvesting roots without destroying the plant.

These and other advantages should be apparent to those skilled in the art from the teachings herein.

Numerous modifications and variations of the present invention are possible and, therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

WHAT IS CLAIMED IS:

1. A process for recovering chemicals from a plant or plant part, comprising:

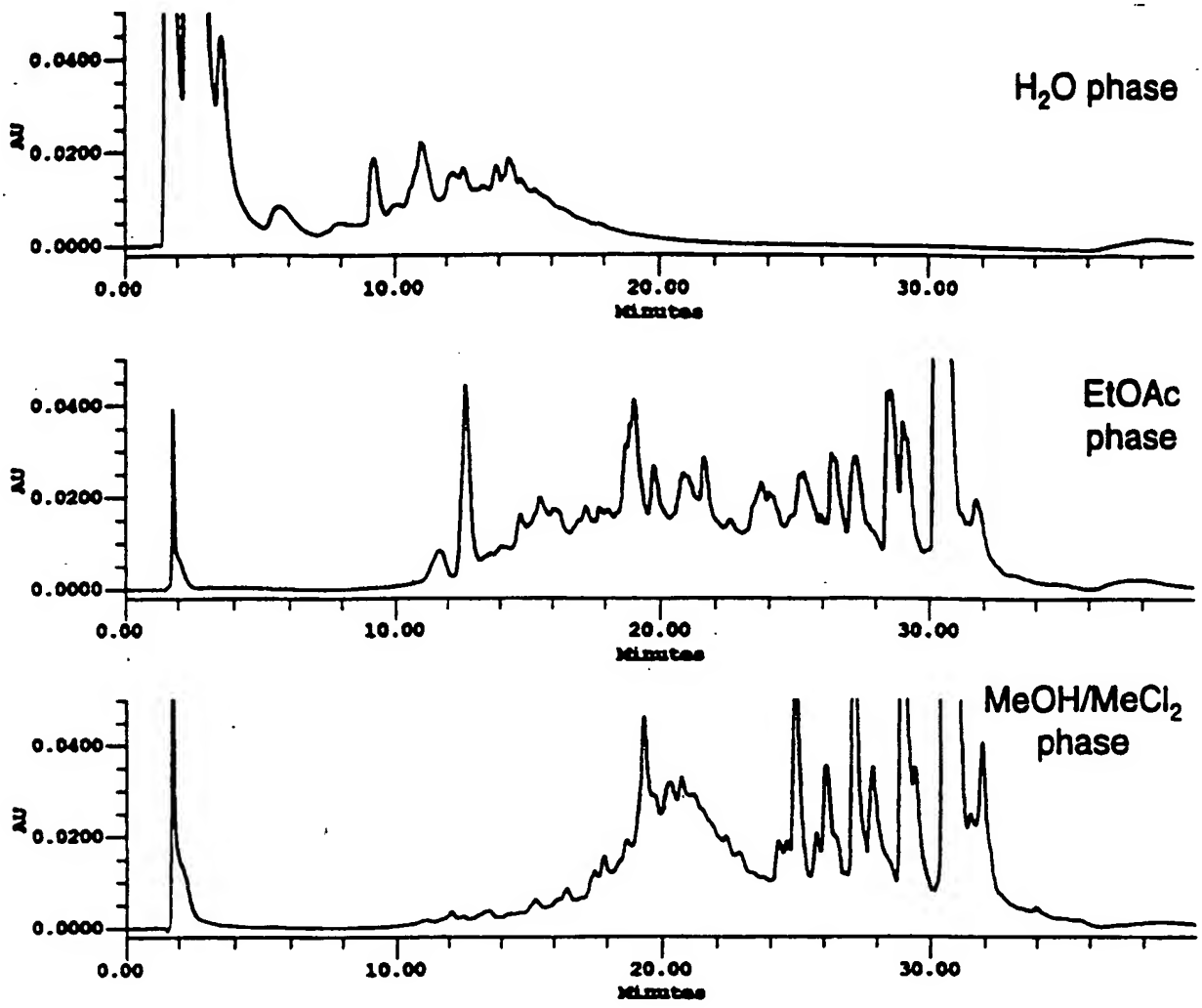
treating a living, intact plant or plant part to induce increased production of at least one chemical in roots of the plant; harvesting roots of the plant, and recovering the at least one chemical compound from harvested roots.

2. The process of Claim 1 wherein the plant or plant part is subjected to a chemical treatment to induce increased production.

3. The process of Claim 1 wherein the plant or plant part is subjected to physical treatment to induce increased production of at least one chemical compound.

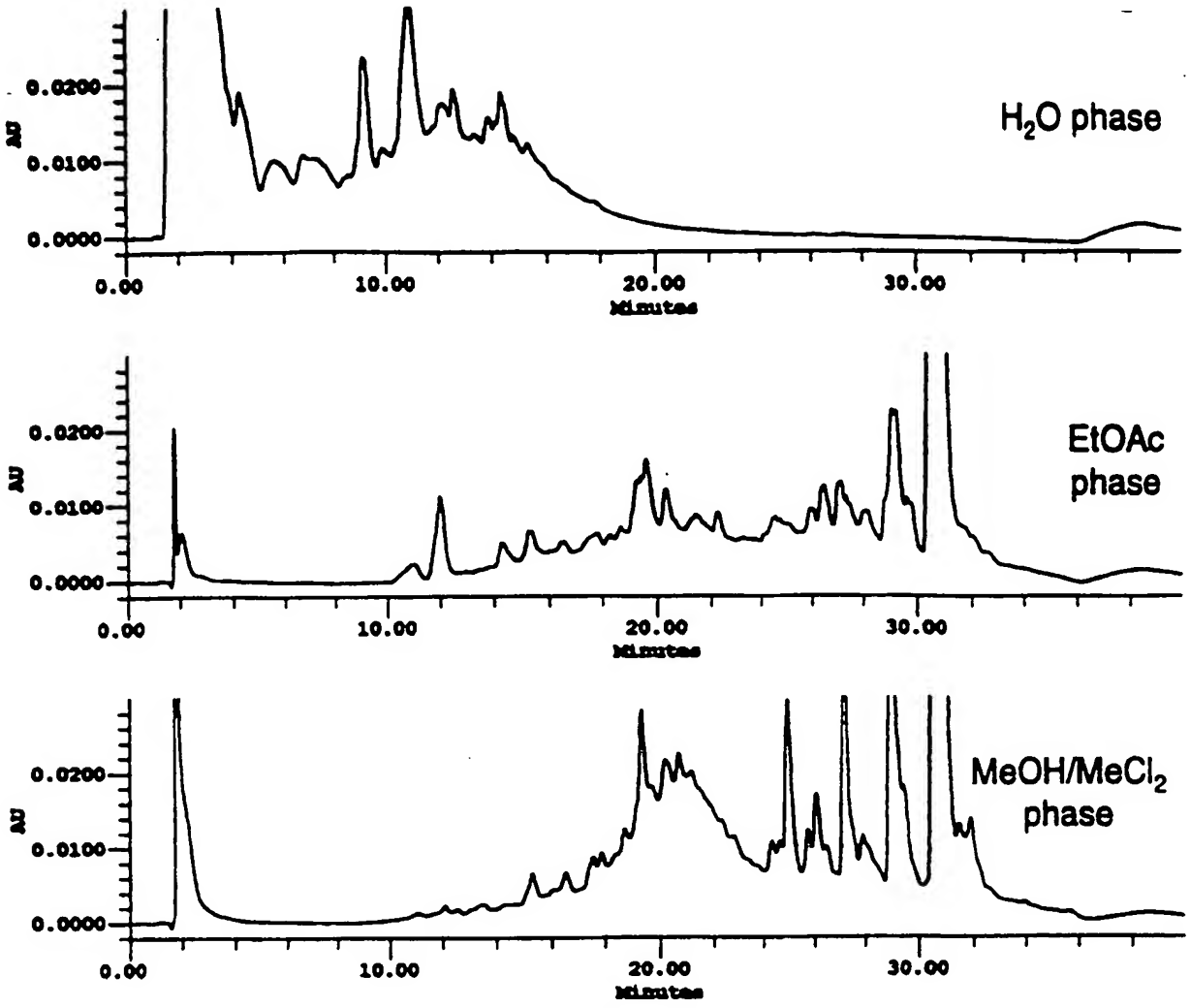
4. A process for generating a library of compounds for subsequent screening, comprising:

forming a library of compounds comprising a plurality of compounds recovered in accordance with the process of Claim 1.



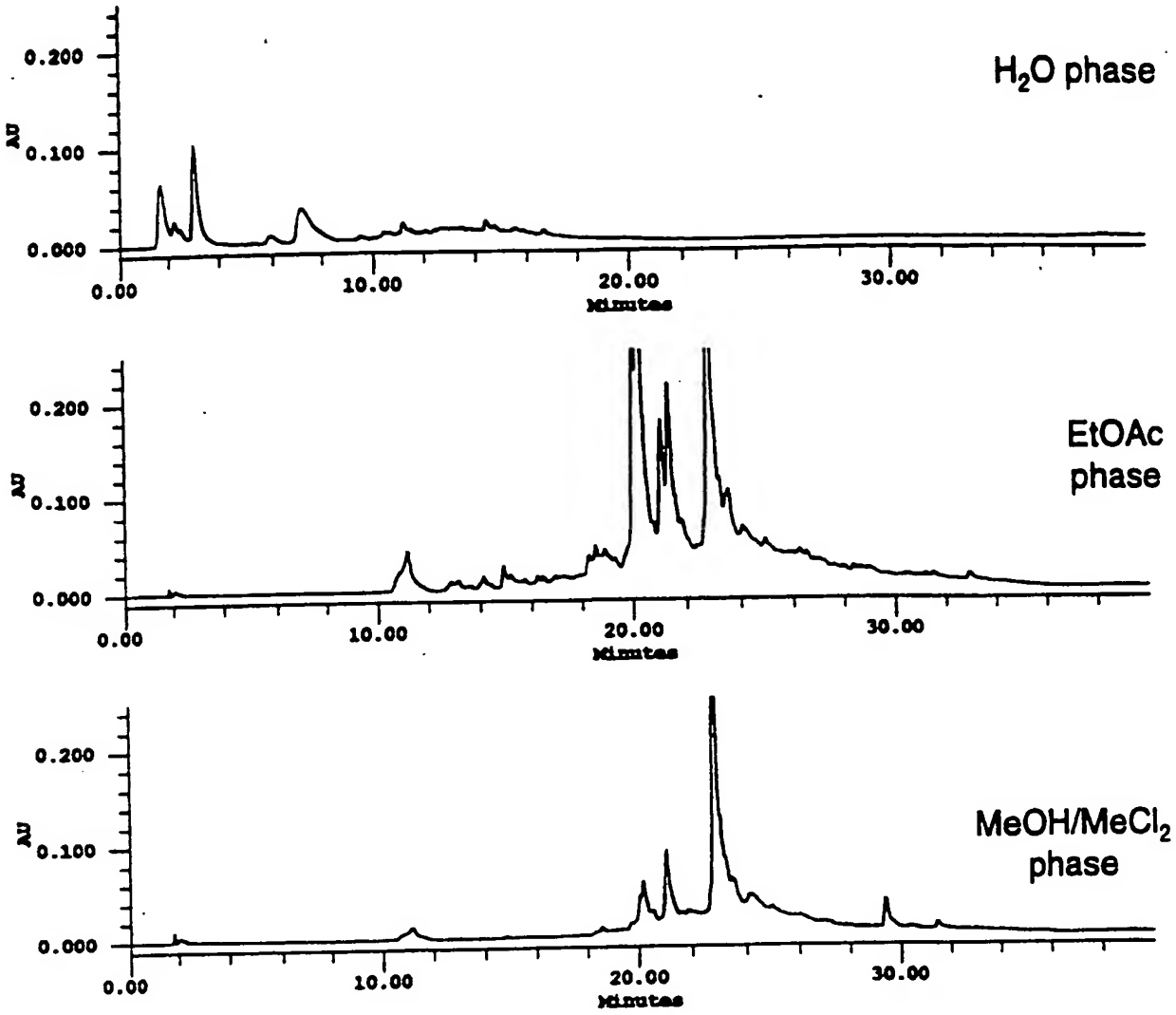
Chemical diversity in different extraction solvents.
Root extracts from *Solanum melongena* (eggplant).
HPLC-profiles with UV detection at 254 nm.

FIGURE 1



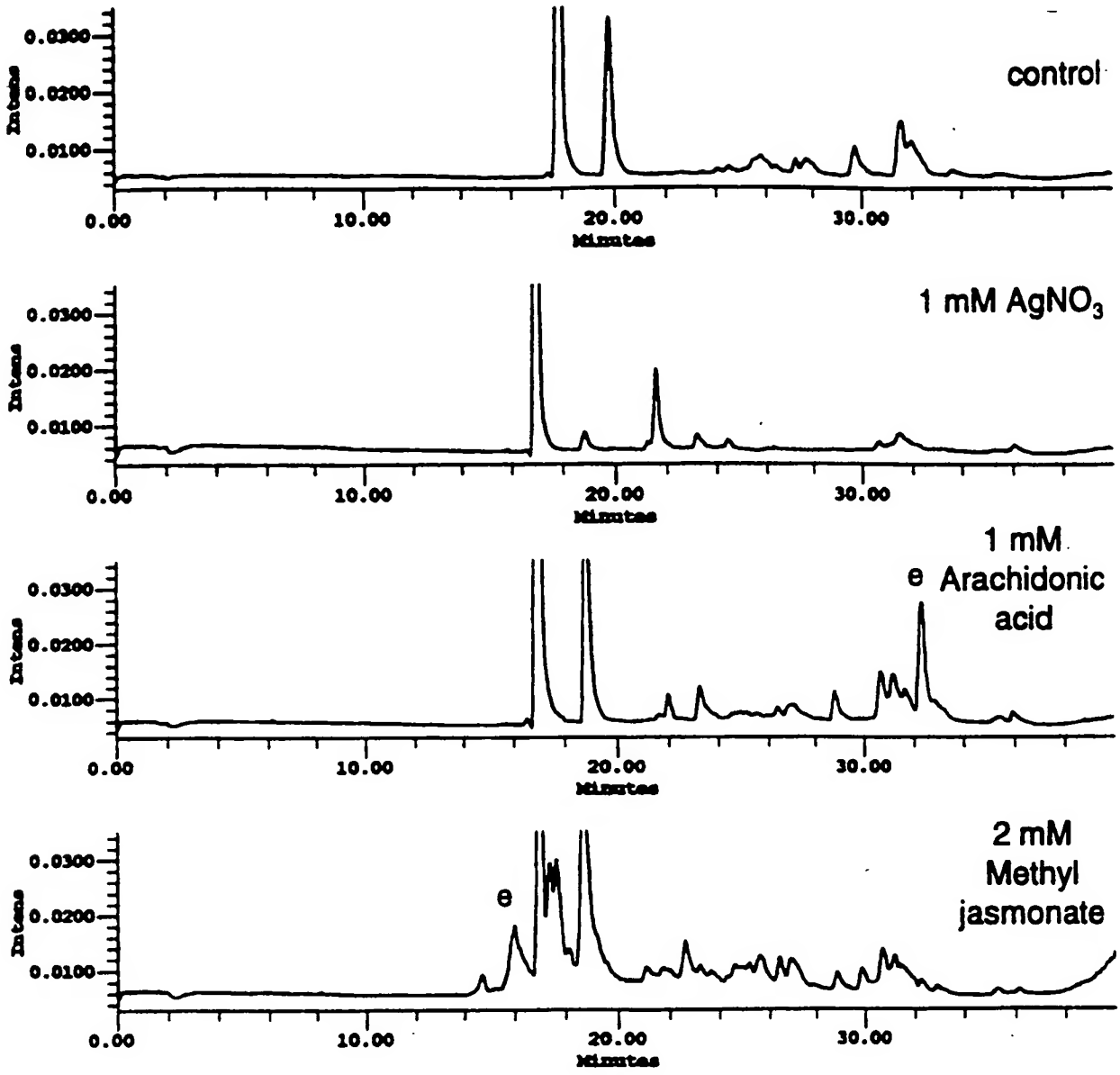
Chemical diversity in different extraction solvents.
Root extracts from *Solanum melongena* (eggplant), elicited with 1 mM Salicylic acid.
HPLC-profiles with UV detection at 254 nm.

FIGURE 2



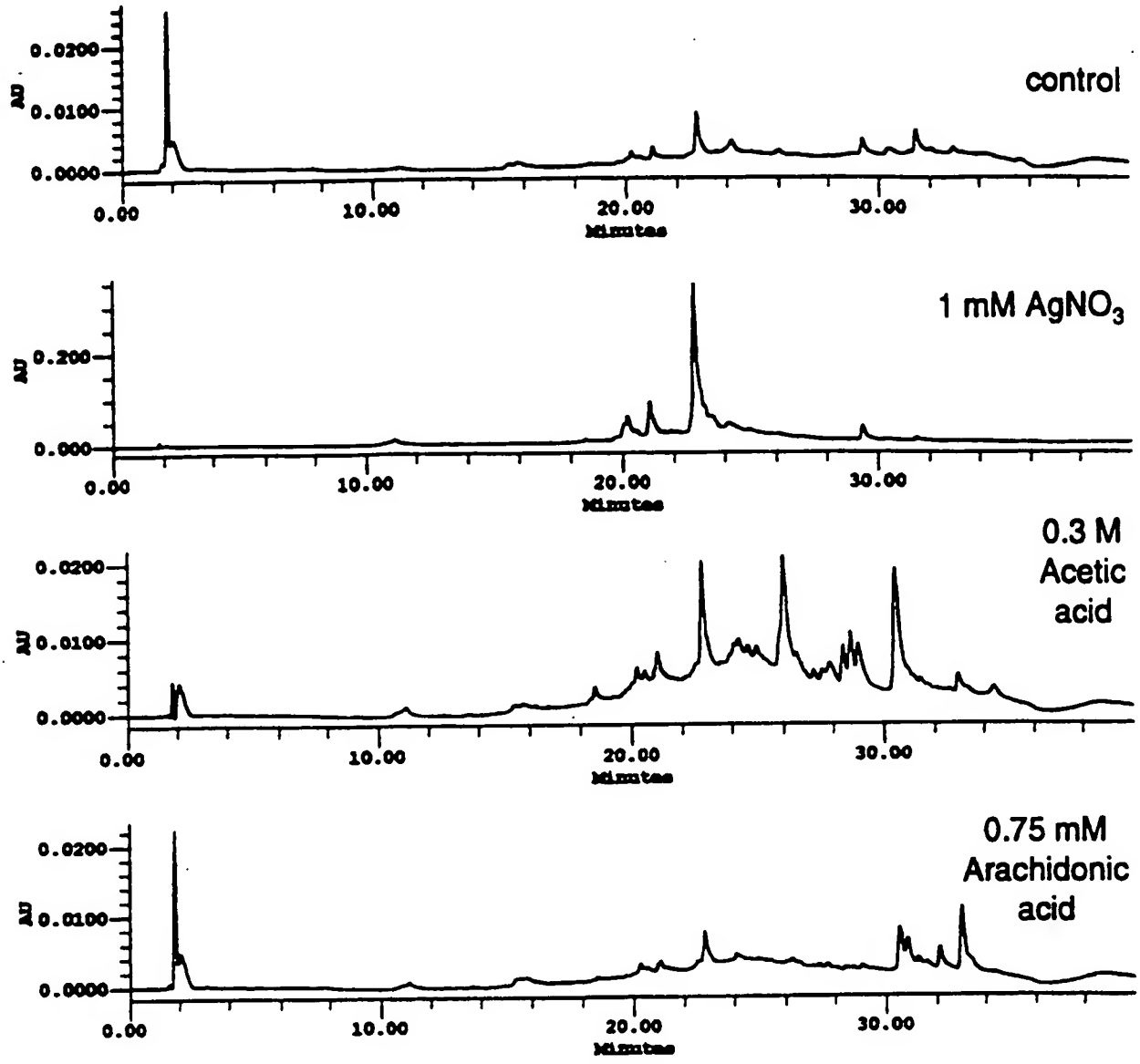
Chemical diversity in different extraction solvents.
Root extracts from *Daucus carota* (carrot), elicited with 1 mM $AgNO_3$.
HPLC-profiles with UV detection at 254 nm.

FIGURE 3



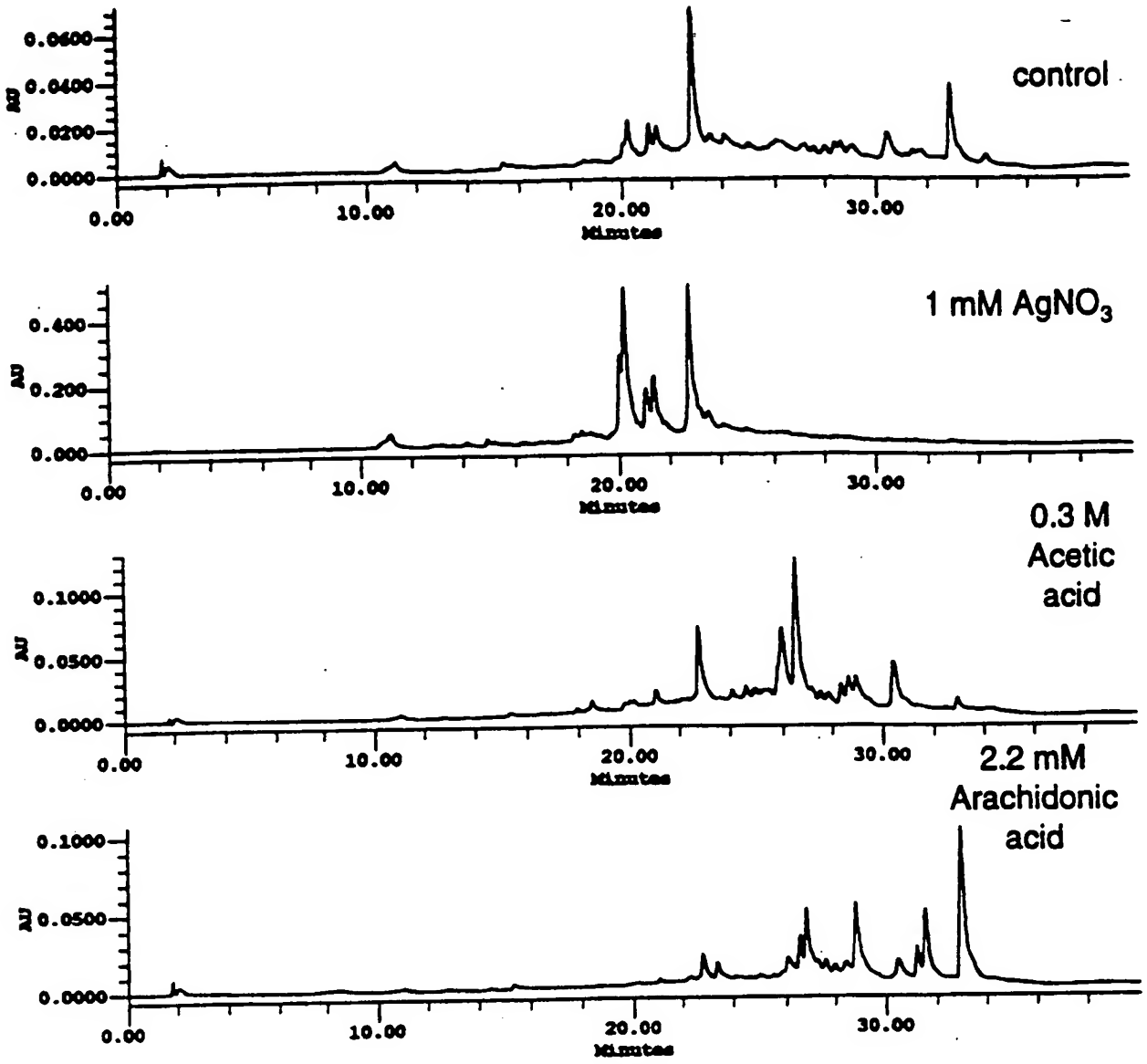
Effect of elicitation on chemical diversity of root extracts.
EtOAc phases of extracts from *Glycyne max* (soybean).
Total Ion Current of chromatograms scanned from 70 m/z to 400 m/z.
e - Elicitor peak

FIGURE 4



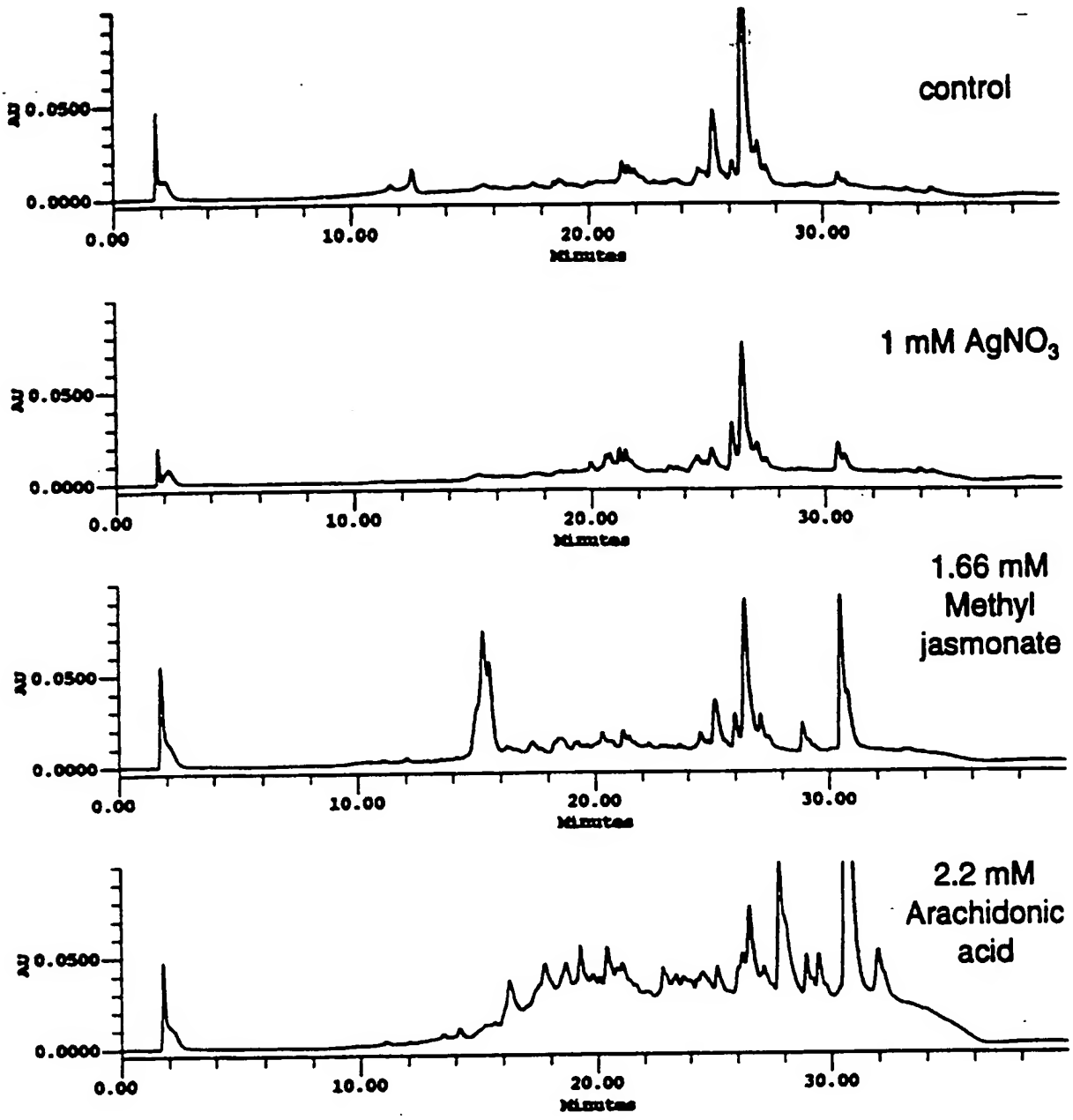
Effect of elicitation on chemical diversity of root extracts. MeOH/$MeCl_2$ phases of extracts from *Daucus carota* (carrot). HPLC-profiles with UV detection at 254 nm.

FIGURE 5



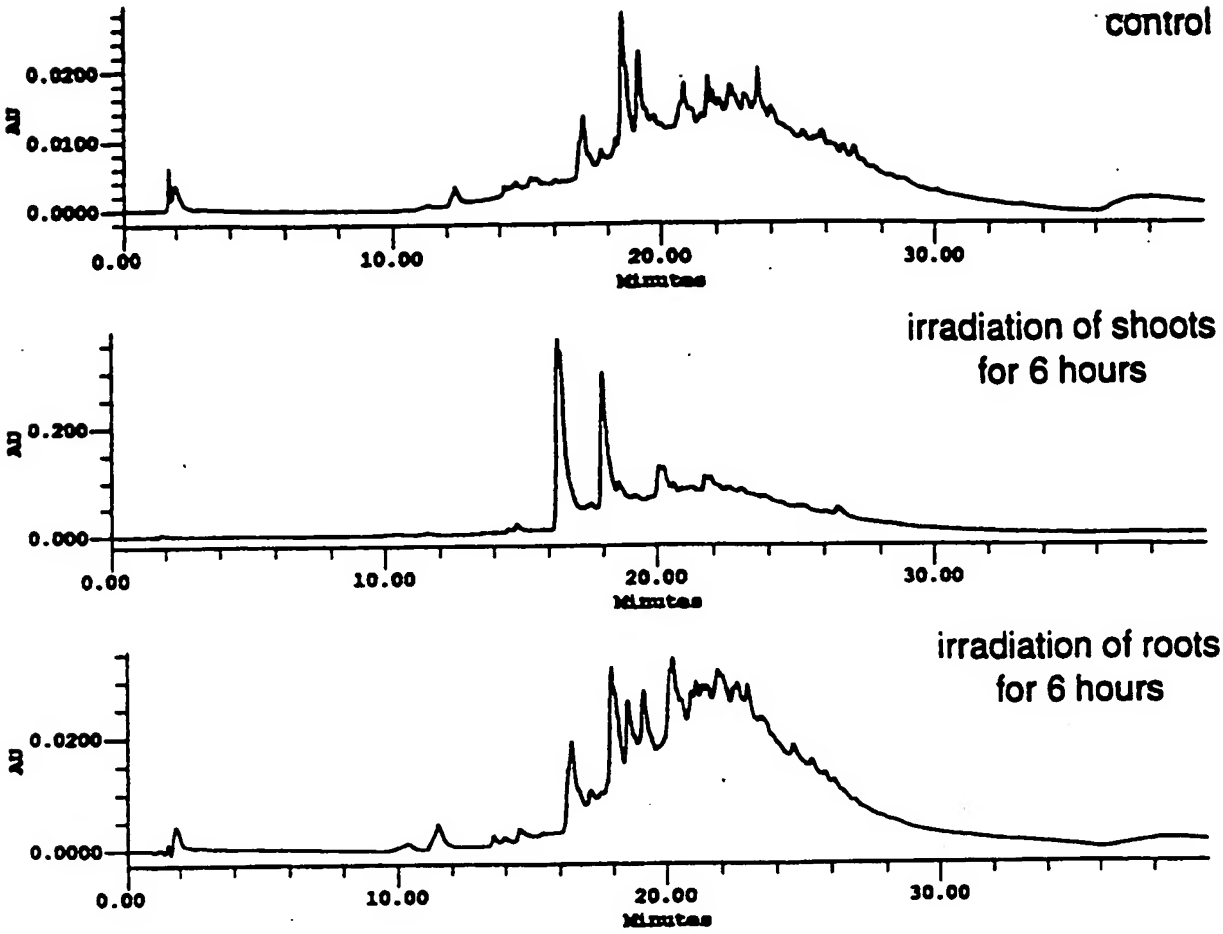
Effect of elicitation on chemical diversity of root extracts.
EtOAc phases of extracts from *Daucus carota* (carrot).
HPLC-profiles with UV detection at 254 nm.

FIGURE 6



Effect of elicitation on chemical diversity of root extracts.
EtOAc phases of extracts from *Lycopersicon aesculentum* (tomato).
HPLC-profiles with UV detection at 254 nm.

FIGURE 7



Effect of UV irradiation on chemical diversity of root extracts.
EtOAc phases of extracts from *Lupinus polyphyllus* (lupine).
HPLC-profiles with UV detection at 254 nm.

FIGURE 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08512

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00
US CL :435/410

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/410

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DANLEY et al. Aflatoxin-induced alteration in the levels of membrane chemicals of subcellular organelles isolated from excised, incubated Bycine max, cv. 'Essex' roots. Mycopathologia. June 1981, Vol. 74, No. 5, pages 149-161, especially Materials and Methods.	1-4
X	NORBERG et al. Phase behaviour and molecular species composition of oat root plasma membrane lipids. Influence of induced dehydration tolerance. Biochemica et Biophysica Acta. 1992, Vol. 1112, pages 52-56, especially Materials and Methods.	1-4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search

10 JULY 1998

Date of mailing of the international search report

04 SEP 1998

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/08512

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAUTER, F-R. Root-specific expression of the LeRse-1 gene in tomato is induced by exposure of the shoot to light. Molecular and General Genetics. 1996, Vol. 252, pages 751-754, especially abstract.	1-4
X	ISOGAI et al. Mikimopine, an opine in hairy roots of tobacco induced by Agrobacterium rhizogenes. Phytochemistry. 1990, vol. 29, No. 10, pages 3131-3134, especially abstract and Figure 4.	1-4